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FANCD2 and CtIP Cooperate to Repair DNA Interstrand Crosslinks

Olga Murina,[1] Christine von Aesch,[1] Ufuk Karakus,[1] Lorenza P. Ferretti,[1] Hella A. Bolck,[1] Kay Hänggi,[1,2] and Alessandro A. Sartori[1,*]
[1]Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057 Zürich, Switzerland
[2]Institute of Experimental Immunology, University of Zurich, Winterthurerstrasse 190, 8057 Zürich, Switzerland
*Correspondence: sartori@imcr.uzh.ch
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SUMMARY

The resolution of DNA interstrand crosslinks (ICLs) requires a complex interplay between several processes of DNA metabolism, including the Fanconi anemia (FA) pathway and homologous recombination (HR). FANCD2 monoubiquitination and CtIP-dependent DNA-end resection represent key events in FA and HR activation, respectively, but very little is known about their functional relationship. Here, we show that CtIP physically interacts with both FANCD2 and ubiquitin and that monoubiquitinated FANCD2 tethers CtIP to damaged chromatin, which helps channel DNA double-strand breaks generated during ICL processing into the HR pathway. Consequently, CtIP mutants defective in FANCD2 binding fail to associate with damaged chromatin, which leads to increased levels of nonhomologous end-joining activity and ICL hypersensitivity. Interestingly, we also observe that CtIP depletion aggravates the genomic instability in FANCD2-deficient cells. Thus, our data indicate that FANCD2 primes CtIP-dependent resection during HR after ICL induction but that CtIP helps prevent illegitimate recombination in FA cells.

INTRODUCTION

Fanconi anemia (FA) is a rare hereditary disorder characterized by bone marrow failure, developmental abnormalities, and cancer predisposition (Moldovan and D'Andrea, 2009). Cells isolated from FA patients display chromosomal instability and hypersensitivity to DNA interstrand crosslink (ICL)-inducing agents such as mitomycin C (MMC) and cisplatin. The high cytotoxicity of MMC, a property exploited in cancer therapy, is primarily based on the strong inhibitory effect of unrepaired ICLs on DNA replication (Deans and West, 2011). Recent studies indicate that the FA pathway orchestrates replication-coupled ICL repair—involving nucleolytic incision, translesion DNA synthesis (TLS), and homologous recombination (HR)—to maintain genomic stability (Knipscheer et al., 2009). In response to ICL damage, the FA core complex, consisting of eight proteins (FANCA, B, C, E, F, G, L, and M), promotes monoubiquitination of FANCD2 and FANCI (Garcia-Higuera et al., 2001; Smogorzewska et al., 2007). The ubiquitinated FANCD2/I complex relocalizes to damaged chromatin, where it coordinates downstream repair events (Kim and D'Andrea, 2012). ICL repair is initiated by nucleolytic incisions on either side of the crosslink and carried out by SLX4-associated XPF-ERCC1 and MUS81-EME1 nucleases and FAN1 (Kottemann and Smogorzewska, 2013). ICL incision converts the stalled replication fork into a one-ended DNA double-strand break (DSB), which is repaired by HR. Interestingly, FA phenotypes can be partially rescued by inhibition of nonhomologous end-joining (NHEJ), suggesting that the FA pathway not only promotes error-free HR but also actively suppresses inappropriate repair of DSB intermediates by NHEJ in order to prevent chromosomal instability (Adamo et al., 2010; Pace et al., 2010).

Although FANCD2 is critically important for ICL repair, its contribution to HR remains largely elusive (Nakanishi et al., 2011; Smogorzewska et al., 2007). HR is initiated by DNA-end resection, which occurs in human cells through the combined action of CtIP and the MRE11-RAD50-NBS1 (MRN) complex, together with DNA2 or EXO1 nucleases (Nimonkar et al., 2011; Sartori et al., 2007). Importantly, DNA-end resection is a key determinant of DSB repair pathway choice, as it commits cells to HR, while, at the same time, suppresses NHEJ (Chapman et al., 2012). A putative connection between the resection machinery and the FA pathway was recently proposed, based on data showing that DNA2 and EXO1 depletion leads to cisplatin hypersensitivity (Karanja et al., 2012). Additionally, CtIP was shown to accumulate at sites of locally induced ICLs (Duquette et al., 2012). However, the contribution of DNA-end resection to ICL repair and the regulation of CtIP by the FA pathway have not yet been thoroughly investigated.

RESULTS

FANCD2 Is Required for CtIP Localization to ICL Damage

Similar to FA cells, we observed that CtIP depletion results in hypersensitivity and increased chromosomal aberrations following MMC treatment, implicating a key role for CtIP in ICL repair (Figures 1A, S1A, and S1B). Because FANCD2 monoubiquitination constitutes a key step of the FA pathway, we investigated

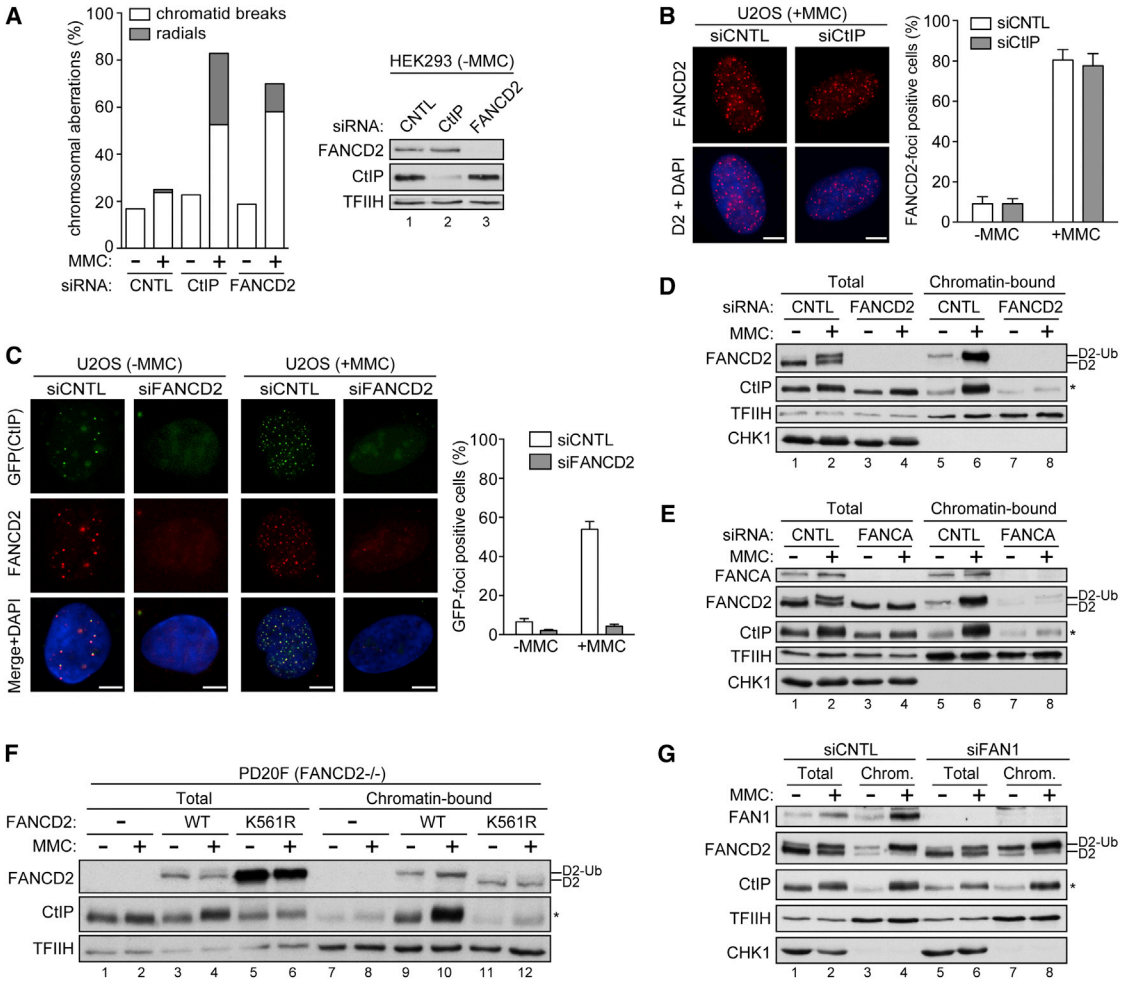

Figure 1. FANCD2 Is Required for the Accumulation of CtIP at Sites of ICL Damage

(A) Metaphase spreads from HEK293 cells transfected with the indicated small interfering RNAs (siRNAs) and treated for 20 hr with MMC (25 ng/ml) were analyzed for chromosomal aberrations. A total of 100 metaphase spreads from three independent experiments were analyzed for each condition. The percentages of cells displaying chromatid breaks or radial chromosomes are shown (see also Figure S1B).

(B) After 48 hr of transfection with indicated siRNAs, U2OS cells grown on coverslips were treated for 24 hr with MMC (120 ng/ml), pre-extracted, fixed, and immunostained for FANCD2. Nuclei were visualized by DAPI-staining. Graph shows the percentage of cells displaying more than ten FANCD2 foci/nuclei.

(C) U2OS cells stably expressing GFP-tagged CtIP were transfected with indicated siRNAs, and 48 hr later, cells grown on coverslips were treated as in (B) and immunostained for FANCD2. Graph shows the percentage of cells displaying more than ten GFP-CtIP foci/nuclei.

(D and E) U2OS cells were transfected with indicated siRNAs, and 48 hr later, cells were treated as in (B) and extracts were analyzed by immunoblotting.

(F) FANCD2 mutant human fibroblasts (PD20F) and PD20F stably expressing wild-type FANCD2 (WT) or K561R mutant FANCD2 were treated as in (B) and harvested for immunoblot analysis (see also Figure S1H).

(G) U2OS cells were transfected with FAN1 siRNA and processed as in (D).

In (B) and (C), for each condition, at least 100 cells were scored and the data are presented as the mean ± SD (n = 3). The scale bar represents 5 μm.

whether CtIP affects FANCD2 foci formation. However, silencing of CtIP did not significantly change the assembly of FANCD2 foci after MMC treatment (Figure 1B). Moreover, FANCD2 monoubiquitination and chromatin assembly still efficiently occurred in CtIP-depleted cells (Figures S1C and S1D). Recently, it was shown that the knockdown of CtIP leads to a 2-fold reduction in FANCD2 accumulation at ICLs induced by 8-methoxypsoralen plus UVA laser microirradiation (PUVA) (Duquette et al., 2012). Therefore, we subjected CtIP-depleted cells to PUVA treatment and noticed partially reduced levels of monoubiquitinated FANCD2 on chromatin (Figures S1E and S1F). Taken together, our data indicate that CtIP is not strictly required for FANCD2 monoubiquitination in response to ICL-inducing agents but that its loss may lead to a negative feedback loop limiting FA pathway activation during ICL repair.

We also noticed that CtIP was highly enriched on chromatin upon MMC or PUVA treatment (Figures S1C and S1F). Consistent with this, ICL damage caused a strong increase in CtIP foci that colocalized with FANCD2 (Figure 1C). Remarkably, both spontaneous and damage-induced CtIP foci were abrogated in FANCD2-depleted cells (Figure 1C). Accordingly, FANCD2 depletion impaired CtIP chromatin association in

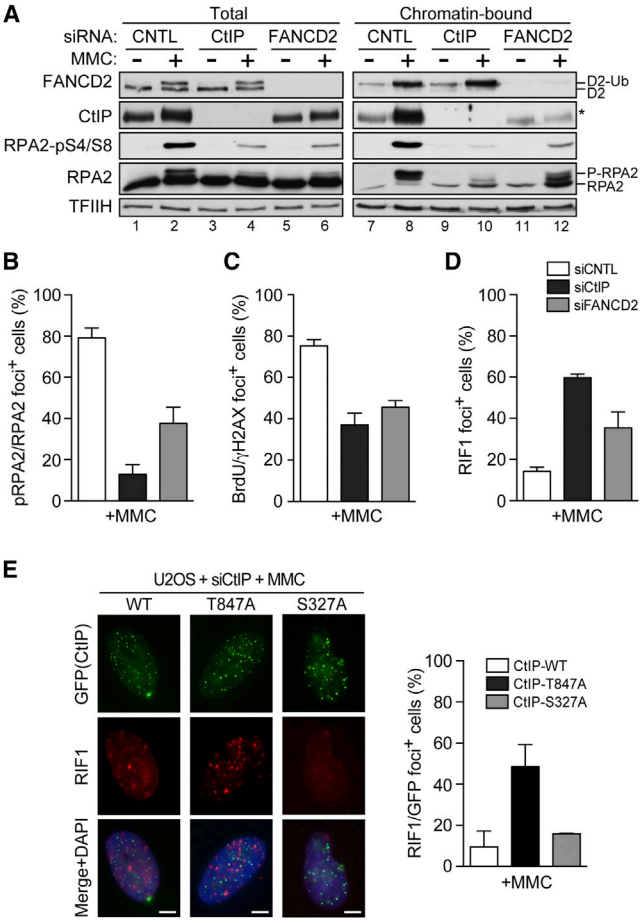

Figure 2. FANCD2 and CtIP Promote DNA-End Resection during ICL Repair

(A) U2OS cells were transfected with indicated small interfering RNAs (siRNAs), and 48 hr after siRNA transfection, cells were either mock-treated or treated for 24 hr with MMC (120 ng/ml) and extracts were analyzed by immunoblotting. RPA2 and P-RPA2 represent nonmodified and hyperphosphorylated forms of RPA2, respectively.

(B) Same cells as in (A) were grown on coverslips, pre-extracted, fixed, and coimmunostained for RPA2-pS4/S8 (pRPA2) and RPA2 (see also Figure S2C). Graph shows the percentage of RPA2-foci-positive cells displaying more than ten pRPA2 foci.

(C) Same cells as in (A) were coimmunostained for γH2AX and BrdU to visualize single-stranded DNA under nondenaturing conditions (see also Figure S2D). Graph shows the percentage of γH2AX-foci-positive cells displaying more than ten BrdU foci.

(D) Same cells as in (A) were immunostained for RIF1 (see also Figure S2E). Graph shows the percentage of nuclei displaying more than ten RIF1 foci.

(E) U2OS cells were transfected with CtIP siRNA, and 24 hr post-siRNA transfection, cells were transfected with siRNA-resistant GFP-tagged CtIP wild-type (WT), T847A, or S327A mutant CtIP (see also Figure S2F). After 48 hr of siRNA transfection, cells were treated as in (A) and immunostained for RIF1. Graph shows the percentage of GFP-foci positive cells displaying more than 10 RIF1 foci. The scale bar represents 5 μm.

In (B)–(E), for each condition, at least 100 cells were scored and data are presented as the mean ± SEM (n = 3).

MMC- and PUVA-treated cells (Figures 1D and S1G). In response to ICL damage, FANCD2 becomes monoubiquitinated at K561 by the FA core complex (Garcia-Higuera et al., 2001; Meetei et al., 2003). Depletion of FANCA, a subunit of the FA core complex, abolished both FANCD2 and CtIP association with damaged chromatin (Figure 1E). Importantly, impaired CtIP accumulation at ICLs in FANCD2-deficient fibroblasts (PD20F) was restored by complementation with wild-type (WT) FANCD2, but not with the K561R mutant (Figures 1F and S1H). In addition, CtIP failed to form MMC-induced foci in cells pretreated with the proteasome inhibitor MG-132, which leads to the sequestration of ubiquitin in the cytoplasm, further indicating that FANCD2 monoubiquitination is a prerequisite for CtIP localization to ICLs (Figure S1I).

During the initial processing of DSBs, CtIP acts together with the MRE11-RAD50-NBS1 (MRN) complex. We therefore explored whether efficient localization of CtIP to ICLs may require the MRN complex. However, while NBS1 downregulation resulted in defective chromatin association of MRE11, the levels of chromatin-bound FANCD2 and CtIP remained unaltered (Figure S1J). On the other hand, monoubiquitinated FANCD2 was reported to directly interact with and recruit FAN1 and SLX4 to coordinate ICL incision (Kottemann and Smogorzewska, 2013; Yamamoto et al., 2011). However, depletion of FAN1 or SLX4 did not significantly affect the binding of CtIP to damaged chromatin (Figures 1G and S1K). Collectively, our results suggest that proper localization of CtIP to ICLs is controlled by FANCD2 but occurs independently of both MRN and structure-specific nucleases involved in ICL incision.

FANCD2 Facilitates CtIP-Mediated DNA-End Resection during ICL Repair

We observed that both MMC and PUVA treatment resulted in robust RPA2-S4/S8 phosphorylation (Figures S2A and S1E). RPA2 phosphorylation, particularly at S4 and S8, has been widely used as a surrogate marker for single-stranded DNA (ssDNA) that is generated by DNA-end resection (Kousholt et al., 2012). Remarkably, knockdown of CtIP or FANCD2 strongly impaired RPA2 hyperphosphorylation in response to ICL-inducing agents, which was particularly evident in the chromatin-bound fractions (Figures 2A and S2B). Likewise, the percentage of cells with RPA2-pS4/S8 foci was significantly reduced in CtIP- or FANCD2-depleted cells (Figures 2B and S2C). Moreover, by immunostaining of cells with anti-bromodeoxyuridine (anti-BrdU), we found that MMC triggered substantial ssDNA formation, which was reduced upon depletion of CtIP or FANCD2 (Figures 2C and S2D). Impaired DNA-end resection commits cells to error-prone repair of DSBs by NHEJ. Recently, RIF1 was characterized as a key NHEJ-promoting factor by virtue of its role in counteracting resection (Chapman et al., 2013; Di Virgilio et al., 2013; Escribano-Díaz et al., 2013; Zimmermann et al., 2013). Indeed, MMC-induced RIF1 foci were elevated in CtIP- or FANCD2-depleted cells, further supporting the idea that both factors promote DNA-end resection and, thus, suppress NHEJ during ICL repair (Figures 2D and S2E).

To further substantiate the role of CtIP-dependent resection in ICL repair, we monitored RIF1 foci in cells expressing GFP-tagged CtIP-WT, CtIP-T847A, or CtIP-S327A (Figure S2F). The mutated residues in CtIP represent CDK phosphorylation sites required for resection (T847A) or for interaction with BRCA1 (S327A) (Figure S2G) (Huertas and Jackson, 2009; Yu and

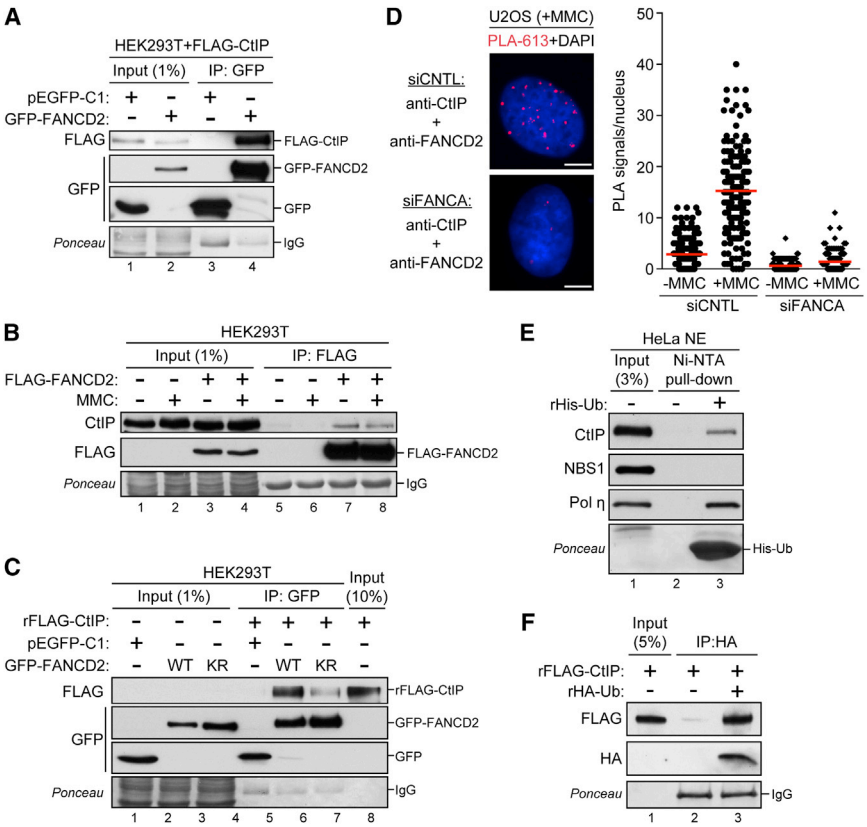

Figure 3. CtIP Physically Interacts with FANCD2 and Ubiquitin

(A) HEK293T cells were cotransfected with FLAG-CtIP along with either empty vector or GFP-FANCD2, and 48 hr after transfection, cells were lysed in NP-40 buffer and whole cell extracts were analyzed before (input) or after immunoprecipitation (IP) with anti-GFP (see also Figure S3A).

(B) HEK293T cells were transfected with either empty vector or FLAG-FANCD2, and 48 hr post-transfection, cells were either mock-treated or treated for 24 hr with MMC (120 ng/ml). Cells were lysed in Triton-X buffer, and whole-cell extracts were analyzed before (input) or after IP using anti-FLAG M2 affinity resin.

(C) HEK293T cells were transfected with empty vector, GFP-FANCD2 (WT), or GFP-FANCD2 K561R (KR). Then 48 hr after transfection, cells were lysed in NP-40 buffer and whole-cell extracts were subjected to IP with anti-GFP. After stringent washings, immunoprecipitated GFP-tagged proteins were incubated with 0.5 μg of recombinant FLAG-tagged CtIP (rFLAG-CtIP; see also Figure S3B). Inputs and recovered protein complexes were analyzed by immunoblotting.

(D) U2OS cells transfected with the indicated siRNAs were left untreated or treated as in (B). After pre-extraction, cells were fixed on coverslips and incubated with antibodies against CtIP and FANCD2 before the detection of protein-protein interactions using a fluorescently labeled probe (PLA-613). Nuclei were visualized by DAPI staining. Graph shows the quantification of the PLA signals/nucleus. PLA signals from at least 100 cells were analyzed (n = 3) (see also Figures S3C and S3D). The scale bar represents 5 μm.

(E) His alone (–) or recombinant His-tagged ubiquitin (rHis-Ub) coupled to nickel nitrilotriacetic acid (Ni-NTA) agarose beads were incubated with HeLa nuclear extracts (NE). Input and precipitated bead fractions from the pull-downs were subjected to immunoblotting. Ponceau staining is shown to indicate the amounts of rHis-Ub used in the pull-down assay.

(F) Recombinant FLAG-tagged CtIP was incubated either alone or together with recombinant hemagglutinin (HA)-tagged ubiquitin and the samples were subjected to IP with anti-HA. Inputs and recovered protein complexes were analyzed by immunoblotting.

Chen, 2004). Our analysis revealed a strong increase in RIF1 foci in MMC-treated T847A mutant cells, whereas the S327A mutation had no significant impact on RIF1 foci formation (Figure 2E). In agreement with this result, T847A mutant cells were hypersensitive to MMC, whereas the viability of cells expressing CtIP-S327A was similar to CtIP-WT cells, further supporting the idea that BRCA1-CtIP interaction is not required for resection during ICL repair (Figures S2H and S2I) (Reczek et al., 2013). Thus far, our findings suggest a pivotal regulatory role for FANCD2 in priming CtIP-mediated DNA-end resection to prevent aberrant NHEJ activity following ICL damage.

CtIP Physically Interacts with FANCD2 and Ubiquitin

Given that CtIP localization to sites of ICL damage is facilitated by FANCD2, we next addressed whether they exist in a complex and found that FLAG-CtIP efficiently coimmunoprecipitated with GFP-FANCD2, and vice versa (Figures 3A and S3A). Moreover, we were able to specifically detect endogenous CtIP coimmunoprecipitating with FLAG-FANCD2 but did not observe significant changes in complex formation upon MMC treatment, suggesting that monoubiquitination of FANCD2 may not be essential for CtIP binding (Figure 3B). In order to further address this issue, we mixed recombinant CtIP purified from insect cells with GFP-FANCD2 immunoprecipitated from human embryonic kidney 293T (HEK293T) cells. While CtIP efficiently bound to FANCD2-WT, it only weakly interacted with the FANCD2-K561R mutant, indicating that FANCD2 and CtIP physically interact and that complex formation might be reinforced by FANCD2 monoubiquitination (Figures 3C and S3B).

To verify the existence of endogenous CtIP-FANCD2 complexes, we performed proximity ligation assays (in situ PLA). As shown in Figure 3D, we could readily detect nuclear PLA signals in undamaged cells, which significantly increased in numbers upon MMC treatment, indicating that the FANCD2-CtIP interaction is enhanced following ICL damage. Moreover, we observed a strong reduction in CtIP-FANCD2 complex formation in FANCA-depleted, but not SLX4- or FAN1-depleted, cells (Figures 3D, S3C, and S3D). These findings further support the importance of FANCD2 monoubiquitination for the accumulation of CtIP at damaged chromatin, presumably by facilitating FANCD2-CtIP interaction. Although sequence analysis revealed that CtIP does not contain any known ubiquitin-binding motifs (Hofmann,

2009), our results prompted us to examine whether CtIP can bind to ubiquitin. As shown in Figure 3E, we discovered that CtIP is able to interact with ubiquitin, although less efficiently compared to Polη that contains a ubiquitin-binding domain (UBD) (Bienko et al., 2005; Plosky et al., 2006). Furthermore, we observed comparable ubiquitin binding abilities of GFP-CtIP and UBZ-domain containing GFP-SLX4 (Figure S3E) (Yamamoto et al., 2011). However, while the ubiquitin I44A mutant completely abolished UBZ-mediated interactions of SLX4 and Polη with ubiquitin, it did not affect the binding of CtIP, implicating a distinct type of ubiquitin recognition (Figure S3F). We were also able to detect an interaction between purified, recombinant proteins, implying that CtIP can directly recognize ubiquitin (Figure 3F). Collectively, these data demonstrate that CtIP interacts with FANCD2 and that FANCD2 monoubiquitination enhances FANCD2-CtIP complex formation, perhaps owing to the ability of CtIP to bind ubiquitin.

FANCD2-CtIP Interaction Promotes Crosslink Resistance

To establish the functional significance of the FANCD2-CtIP interaction for ICL repair, we sought to identify the FANCD2-binding motif in CtIP. Whereas FANCD2 did not bind to a region of CtIP containing putative coiled-coil motifs (45–160), it bound efficiently to fragments of CtIP comprising amino acid (aa) residues 45–298 or 45–371 (Figure 4A), highlighting the region between aa residues 160–298 of CtIP to be important for FANCD2 binding. Detailed protein sequence analysis of this region revealed four motifs with high sequence conservation between vertebrates (Figure S4A). Remarkably, cells expressing RRK/AAA or RYIE/AAIA mutant variants of CtIP were impaired in MMC-induced GFP-CtIP foci formation and showed increased RIF1 foci (Figures S4B and S4C). Consistent with a defect in CtIP foci formation, RRK/AAA and RYIE/AAIA mutants exhibited reduced FANCD2 binding in glutathione S-transferase (GST) pull-down experiments (Figures 4B and 4C). Moreover, PLA signals were reduced in both CtIP mutants after MMC treatment, further supporting the role of RRK and RYIE motifs in CtIP-FANCD2 interaction (Figures 4D and 4E). MMC-induced CtIP foci were also abrogated in U2OS cells stably expressing GFP-tagged CtIP-RRK/AAA and -RYIE/AAIA mutants, whereas RIF1 foci were significantly increased in those cells (Figure 4F). However, both CtIP mutants were efficiently recruited to DSB-containing tracks generated by laser microirradiation (Figure S4D). Underscoring a differential regulation of CtIP in response to ICL damage, CtIP recruitment to laser-induced DNA lesions was FANCD2-independent (Figure S4E). Of note, a truncated CtIP mutant lacking residues 153–322 was still proficient in ubiquitin binding, indicating that neither RRK (aa 177–179) nor RYIE (aa 185-188) sequence motifs are required for the interaction between CtIP and ubiquitin (Figures S4F and S4G). Finally, both RRK/AAA and RYIE/AAIA mutant cells were hypersensitive to MMC, further supporting the idea that FANCD2 regulates CtIP functionality during ICL repair (Figure 4G).

CtIP Counteracts ICL-Induced DNA Damage in the Absence of FANCD2 Activation

To genetically determine the epistatic relationship between CtIP and FANCD2 in ICL repair, we generated an MRC5 cell line stably expressing doxycycline (DOX)-inducible small hairpin RNA (shRNA) against CtIP (Figure 5A). Interestingly, depletion of FANCD2 in DOX-treated MRC5 cells led to a further increase in MMC hypersensitivity (Figures 5A and 5B). In agreement with this, survival of PD20 cells upon MMC treatment was further reduced after silencing of CtIP (Figure S5A). CtIP/FANCD2-deficient MRC5 cells also showed elevated levels of MMC-induced RIF1 foci and radial chromosomes compared to cells depleted for either factor alone, indicative of potent, illegitimate repair of DSBs by NHEJ (Figures 5C, 5D, S5B, and S5C). Previously, BRCA1 has been reported to regulate the accumulation of FANCD2 into repair foci and CtIP recruitment to PUVA-induced ICLs (Garcia-Higuera et al., 2001; Duquette et al., 2012). Further supporting a dual, nonredundant role for CtIP in ICL repair, knockdown of BRCA1 in DOX-treated MRC5^{shCtIP} cells resulted in increased MMC sensitivity (Figure S5D). Thus, our data suggest that CtIP-dependent DNA-end resection is essential to counteract the toxic effects of ICL damage when the FA/BRCA signaling pathway is compromised.

ATR kinase is a major regulator of the FA pathway and promotes FANCD2/I monoubiquitination (Andreassen et al., 2004; Smogorzewska et al., 2007). Recently, both ATM and ATR kinases have been implicated in DNA-damage-induced CtIP phosphorylation (Peterson et al., 2013; Wang et al., 2013). In order to gain further mechanistic insight into the regulation of CtIP during ICL repair, we applied selective ATR and/or ATM inhibitors prior to MMC treatment (Reaper et al., 2011). CtIP and RPA2 phosphorylation was strongly elevated after ATR inhibition; meanwhile, FANCD2 monoubiquitination and CHK1 phosphorylation were reduced as expected (Figure 5E, lane 2). Interestingly, MMC-induced hyperphosphorylation of CtIP and, to a lesser extent, of RPA2 was reversed when both inhibitors were combined (Figure 5E, lane 4). These data suggest that ATM gets hyperactivated when ATR kinase is blocked, probably as a result of prevalent cleavage of collapsed replication forks into DSBs. The structure-specific endonuclease MUS81-EME1 has been implicated in the conversion of stalled replication forks into DSBs, particularly in checkpoint-deficient cells (Hanada et al., 2006; Murfuni et al., 2013). Indeed, inhibition of ATR combined with MUS81 depletion significantly reduced the phosphorylation of H2AX and KAP1, both established ATM targets, indicating that MUS81 is at least partially required for the processing of ICL-stalled forks into DSBs (Figure 5F). Remarkably, phosphorylation levels of CtIP were also decreased, further implying CtIP in promoting DNA-end resection and HR of replication-associated DSBs in the absence of a fully functional FA pathway. Taken together, our results demonstrate that during conventional ICL repair, CtIP-mediated DNA-end resection is regulated by FANCD2 but that CtIP also helps prevent illegitimate repair of stalled forks in FA-pathway-defective cells.

DISCUSSION

CtIP is an essential factor required for the initiation of DNA-end resection during HR and, thus, for the suppression of DSB repair by NHEJ. Given that FA-pathway-deficient cells exhibit increased chromosomal instability in response to ICL damage, FA proteins have also been implicated in NHEJ inhibition. Indeed, we

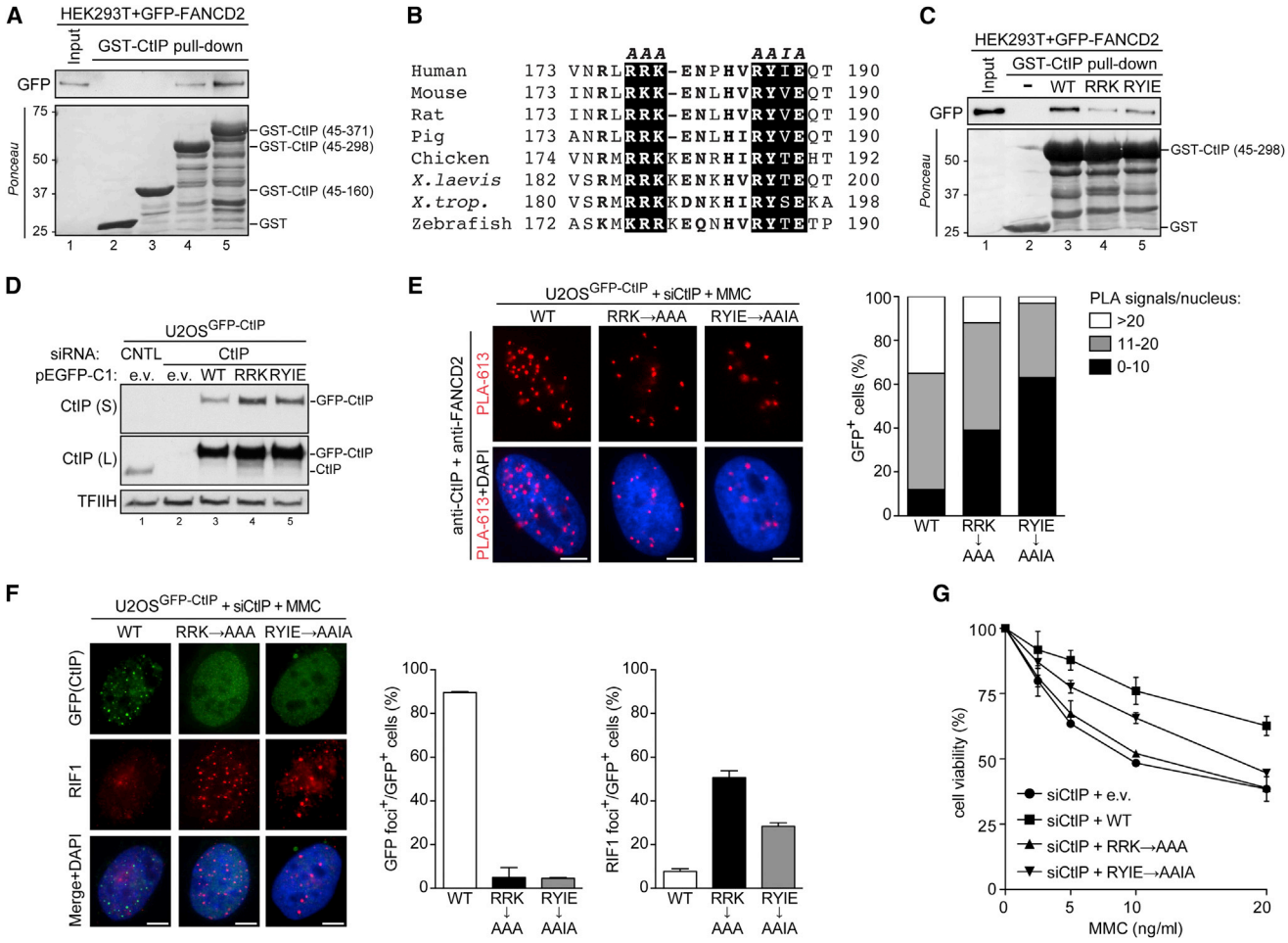

Figure 4. Functional Characterization of CtIP Mutants Impaired in FANCD2 Binding

(A) GST or GST-CtIP proteins were coupled to glutathione Sepharose beads and incubated with HEK293T cell lysates transiently overexpressing GFP-FANCD2. The recovered materials were analyzed by immunoblotting.

(B) Alignment of the putative FANCD2-interacting region in CtIP orthologs. RRK and RYxE motifs are highlighted in black boxes. Other, highly conserved amino acid residues are marked in bold typeface (see also Figure S4A).

(C) GST or indicated GST-CtIP (45–298) proteins were coupled to glutathione Sepharose beads and incubated with HEK293T cell lysates transiently overexpressing GFP-FANCD2. The recovered materials were analyzed by immunoblotting.

(D) U2OS cells stably expressing siRNA-resistant GFP-tagged wild-type (WT) and mutant (RRK/AAA and RYIE/AAIA) CtIP were transfected with CtIP siRNA for 72 hr, and whole-cell extracts were analyzed by immunoblotting. (S) and (L) indicate short and long exposures of the same immunoblot, respectively.

(E) Same cells as in (D) were transfected with CtIP siRNA. After 48 hr of siRNA transfection, cells grown on coverslips were treated for 24 hr with MMC (120 ng/ml), fixed, and incubated with antibodies against CtIP and FANCD2 before the detection of protein-protein interactions using a fluorescently labeled probe (PLA-613). Graph shows the quantification of the PLA signals/nucleus in GFP-positive cells. PLA signals from at least 100 cells were analyzed (n = 2).

(F) Same cells as in (E) were fixed and immunostained for RIF1. Graphs show the percentage of GFP-positive cells displaying more than ten GFP-CtIP foci or more than ten RIF1 foci, respectively. For each condition, at least 100 cells were scored. Data are presented as the mean ± SEM (n = 2).

(G) Same cells as in (D) were either mock-treated or continuously treated with the indicated doses of MMC, and the survival was determined after 5 days using the CellTiter-Blue cell viability assay. Data are presented as the mean ± SD (n = 3).

In (E) and (F), the scale bar represents 5 μm.

observed that depletion of CtIP or FANCD2 leads to an increase in radial chromosome formation and RIF1 foci after MMC treatment, indicative of enhanced NHEJ activity. Accordingly, DNA-end resection, as measured by phosphorylated RPA2 and BrdU focus formation, was reduced in CtIP- or FANCD2-depleted cells. Several mechanisms have been proposed how FA proteins prevent NHEJ, including the restriction of NHEJ factors to access DNA termini, cryptic exonuclease, or nucleosome-assembly activity of FANCD2 (Adamo et al., 2010; Pace et al., 2010; Sato et al., 2012). Based on our data, we propose that FANCD2 suppresses error-prone NHEJ through promoting CtIP-dependent resection (Figure S6).

We observed that monoubiquitinated FANCD2 tethers CtIP to damaged chromatin and identified two short, highly conserved motifs within CtIP responsible for CtIP-FANCD2 interaction. Consequently, CtIP mutants defective in FANCD2 binding are

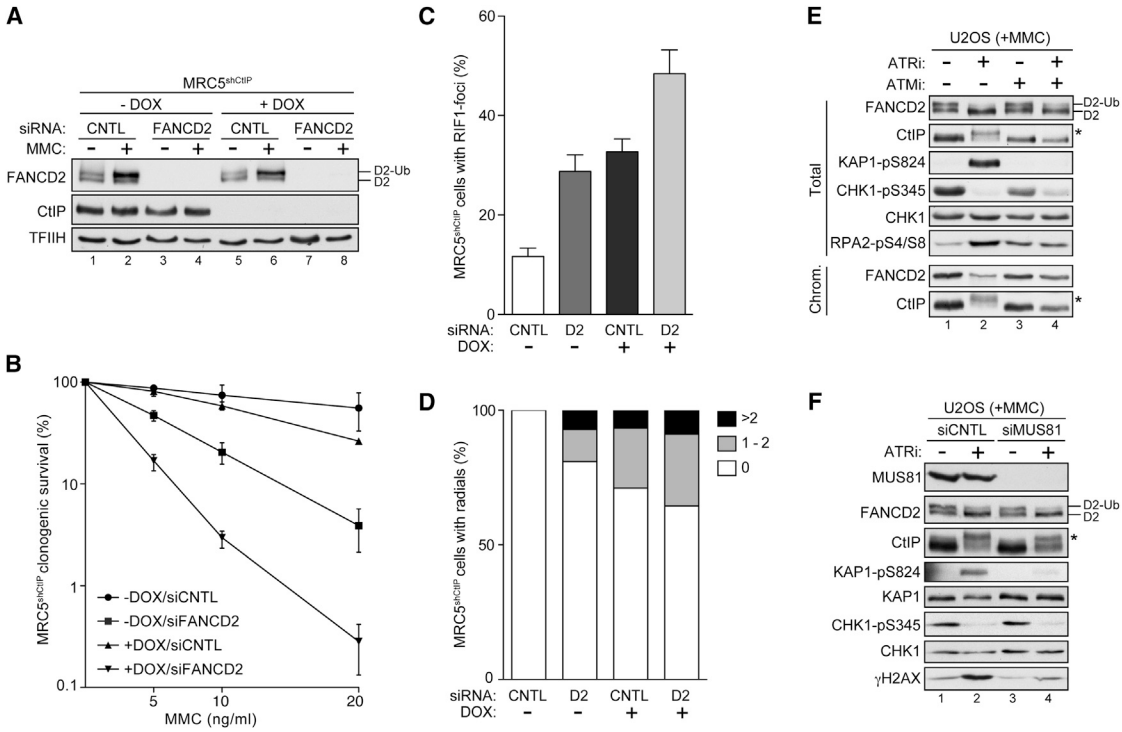

Figure 5. CtIP Contributes to Genome Stability in FA-Pathway-Deficient Cells

(A) MRC5 cells stably expressing doxycycline (DOX)-inducible shRNA against CtIP (MRC5^{shCtIP}) were transfected with indicated siRNAs. Then 24 hr after siRNA transfection, cells were cultivated in the absence or presence of DOX (1 μg/ml) for 48 hr. Cells were treated with MMC (120 ng/ml) for 24 hr and subjected to immunoblotting.

(B) Same cells as in (A) were treated for 24 hr with indicated doses of MMC, and survival was determined after 10 days by colony-formation assay. Data are presented as the mean ± SD (n = 3).

(C) Same cells as in (A) were immunostained for RIF1. Graph shows the percentage of RIF1-foci-positive cells displaying more than ten RIF1 foci. For each condition, at least 100 cells were scored. Data are presented as the mean ± SD (n = 3) (see also Figure S5B).

(D) Metaphase spreads from the same cells as in (A) treated for 20 hr with MMC (20 ng/ml) were analyzed for chromosomal aberrations. A total of 45 metaphase spreads from three independent experiments were analyzed for each condition. The percentages of cells displaying radial chromosomes are shown (see also Figure S5C).

(E) U2OS cells were pretreated for 15 min with DMSO, ATR inhibitor (VE-821, 1 μM), ATM inhibitor (KU-55933, 10 μM), or both inhibitors together. Cells were then treated for 20 hr with MMC (120 ng/ml), and extracts were analyzed by immunoblotting.

(F) U2OS cells were transfected with MUS81 siRNA, and 48 hr after siRNA transfection, cells were pretreated for 15 min with DMSO or ATR inhibitor (VE-821, 1 μM). Cells were then treated as in (E) and harvested for immunoblot analysis.

impaired in the formation of MMC-induced foci. Whereas FANCD2 monoubiquitination may not be essential for the physical interaction with CtIP, it is required to retain CtIP on damaged chromatin. Remarkably, we show that CtIP directly interacts with ubiquitin in vitro. Although bioinformatic analysis failed to predict any motifs resembling known UBDs in CtIP, it is plausible that the FANCD2-CtIP interaction is reinforced by the ability of CtIP to recognize ubiquitin. However, a CtIP mutant lacking both FANCD2-interacting motifs was proficient in ubiquitin binding. Furthermore, a mutant form of ubiquitin (I44A) defective in most UBD-mediated interactions was still able to interact with CtIP. Therefore, one could hypothesize that CtIP may employ a dual mode of recognizing monoubiquitinated FANCD2, but, if so, binds ubiquitin by a unique mechanism that involves a new type of UBD (Bomar et al., 2007). Clearly, further investigations are needed to establish the role of CtIP-ubiquitin interaction in the DNA damage response. On the other hand, it was proposed that monoubiquitination of FANCD2 could alter FANCD2 conformation (Joo et al., 2011). Thus, it is tempting to speculate that such structural changes in FANCD2 stimulate CtIP-FANCD2 interaction. Finally, the FANCD2/I complex may also facilitate CtIP recruitment to damaged chromatin via its histone chaperone activity (Sato et al., 2012). Increased MMC-induced sensitivity and RIF1 foci formation in cells expressing CtIP mutants (RRK/AAA and RYIE/AAIA) further strengthen the significance of CtIP-FANCD2 interaction in ICL repair. Notably, R177Q and Y186C, two cancer-associated missense mutations in human CtIP, map exactly to the region implicated in FANCD2 interaction (Figure S4A). Given that RRK and RYIE motifs reside in a highly conserved stretch of 12 amino acids, it is also reasonable to think that they constitute a single FANCD2 interaction "domain." These data are similar to those described in the accompanying paper by Unno et al. (2014) published in this issue of *Cell Reports*.

Depletion of CtIP in FANCD2-deficient cells aggravates the phenotypes of cells lacking either factor alone, indicating that CtIP contributes to genome stability in FA-pathway-defective

cells. Likewise, inactivation of FAN1 or SLX4 enhanced crosslink sensitivity of cells compromised in FANCD2 activation, suggesting that these proteins, though being recruited to ICLs by FANCD2, can promote MMC resistance in a FA-pathway-independent manner (Yamamoto et al., 2011; Zhou et al., 2012). Furthermore, only partial epistasis of FANCD2 over CtIP could be explained by an additional role of CtIP upstream of FANCD2 or by the proposed role of FANCD2 in protecting stalled forks from degradation (Duquette et al., 2012; Schlacher et al., 2012). There is increasing evidence that arrested and unprotected replication forks frequently collapse and give rise to DSBs, which can then undergo resection and repair by HR (Couch et al., 2013). Accordingly, we observed enhanced ATM-dependent phosphorylation of CtIP and RPA2 in cells cotreated with MMC and ATR inhibitor, indicative of ongoing DSB resection (Fugger et al., 2013). In line with this, CtIP phosphorylation was reduced upon depletion of MUS81, an endonuclease implicated in the cleavage of stalled forks and DSB formation (Ciccia et al., 2008). Furthermore, CtIP-dependent processing of collapsed forks upon hydroxyurea treatment was recently reported to be beneficial for genome integrity in the absence of FANCM (Blackford et al., 2012). Therefore, we conclude that CtIP can partially suppress genomic instability in the absence of FANCD2, whereas, in FA-pathway-proficient cells, monoubiquitinated FANCD2 coordinates CtIP-mediated DNA-end resection during ICL repair.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

U2OS, HEK293T, and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. U2OS clones stably expressing siRNA-resistant forms of GFP-CtIP were described previously (Sartori et al., 2007). FANCD2-deficient cells (PD20F) were obtained from Fanconi Anemia Research Foundation (FARF) and cultured in DMEM supplemented with 10% FCS and standard antibiotics. PD20F cells complemented with FANCD2 wild-type (FARF) or K561R (kindly provided by Josef Jiricny) were grown in standard medium supplemented with 1 μg/ml Puromycin. MRC5^{shCtIP} cells were grown in DMEM supplemented with 10% Tet system approved FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, Blasticidin (5 μg/ml), and Zeocin (250 μg/ml). Plasmids were transfected either by using the standard calcium phosphate method or FuGENE 6 (Roche) according to manufacturer's instructions. Transfection of all siRNA oligos was done with 40 nM final concentration using Lipofectamine RNAiMAX (Invitrogen). Data for survival curves were generated by colony-formation assays as described previously (Sartori et al., 2007).

Triton Extraction

Isolation of Triton-insoluble (chromatin-enriched) fraction was performed as previously described (Peña-Diaz et al., 2012). In brief, cells were rinsed twice in cold PBS and incubated for 5 min on ice in preextraction buffer (25 mM HEPES [pH 7.4], 50 mM NaCl, 1 mM EDTA, 3 mM $MgCl_2$, 300 mM sucrose, 0.5% Triton X-100, and protease inhibitors). After buffer removal, adherent cellular material was harvested by scraping the cells into Laemmli buffer. The chromatin-enriched fraction was then heat denatured, sonicated, and analyzed by immunoblotting.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.069.

AUTHOR CONTRIBUTIONS

O.M. and A.A.S. designed the research. L.P.F. performed the laser microirradiation experiments, and H.A.B. helped with the ubiquitin binding assay. K.H. generated the inducible MRC5^{shCtIP} cell line. All other experiments were performed by O.M. with the help of C.A. and U.K. O.M. and A.A.S. analyzed the data and wrote the paper.

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